EXHIBIT 5

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Isolation of New Temperature-Sensitive Mutants of *Saccharomyces cerevisiae* Deficient in Mannose Outer Chain Elongation

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We have isolated two temperature-sensitive *Saccharomyces cerevisiae* mutants which exhibit a deficiency in mannose outer chain elongation of asparagine-linked oligosaccharide. The size of yeast glycoprotein, secretory form of invertase, of one mutant (*och1*) was slightly larger than that of the *sec18* mutant at the non-permissive temperature, while that of the other mutant (*och2*) was almost the same as that of the *sec18* mutant. Unlike *sec* mutants, the *och* mutants were not deficient in secretion of invertase. The *och1* mutant showed a 2+:2− cosegregation with regard to the temperature sensitivity and mannose outer chain deficiency, suggesting that a single gene designated as *OCH1* is responsible for these two phenotypes. The *och1* mutant stopped its growth at the early stage of bud formation and rapidly lost its viability at the non-permissive temperature. The *och1* mutation was mapped near the *ole1* on the left arm of chromosome VII. The *och1* mutant cells accumulated the external invertase containing a large amount of core-like oligosaccharides ($Man_{9-10}GlcNAc_2$) and a small amount of high mannose oligosaccharides ($> Man_{50}GlcNAc_2$) at the non-permissive temperature. Production of the active form of human tissue-type plasminogen activator was increased in the *och1* mutant compared with the parental strain, suggesting the potential advantage of this mutant for the production of mammalian-type glycoproteins which lack mannose outer chains in yeast.

KEY WORDS — Protein glycosylation; *Saccharomyces cerevisiae*; outer chain mannosylation.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a valuable host for the expression of heterologous genes, because yeast has a glycosylation system resembling the one in higher eukaryotes. The asparagine-linked (N-linked) glycosylation pathways are similar in yeast and mammalian cells up to the first stages of glycan processing, although the later stages of carbohydrate chain processing are different. In mammalian cells, the common lipid-linked precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$, which is transferred to protein, can be processed partially to yield the high mannose species $Man_{5-9}GlcNAc_2$ or more completely to provide oligosaccharides with a $Man_3GlcNAc_2$ core containing peripheral N-acetylglucosamine, galactose, sialic acid, or fucose residues (Hubbard and Ivatt, 1981). In *S. cerevisiae*, on the other hand, only three glucose and one mannose residues are removed from the common precursor oligosaccharide and a subsequent elongation takes place through a stepwise addition of mannose residues to form outer chains consisting of a poly-α1,6-Man backbone with side chains of mannose in α1,2-, α1,3-, and phosphodiester linkages (Cohen *et al.*, 1982; Kukuruzinska *et al.*, 1987; Tanner and Lehle, 1987; Hernandez *et al.*, 1989a,b).

Yeast has advantages in mutant isolation and genetic manipulation, and offers the possibility of mutant constructions that to date are not available in higher eukaryotes. Several yeast mutants defective in a certain part of the glycosylation pathway have been isolated so far. Huffaker and Robbins (1982, 1983) reported seven *alg* (asparagine-linked glycosylation) mutants, which were defective in lipid-linked oligosaccharide biosynthesis. Several

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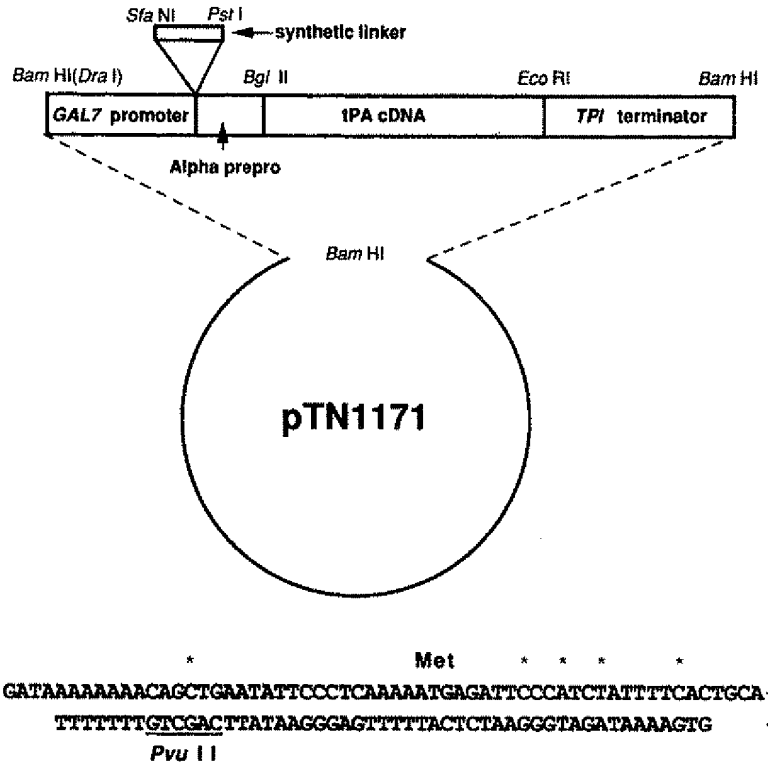

Figure 1. (A) The structure of pTN1171, tPA expression vector for yeast. The cDNA of mature tPA, fused to the α-factor prepro region, was placed at the 3' end of the *GAL7* promoter via the synthetic linker oligonucleotides. The whole expression unit, indicated as a bar in the figure, was introduced into the yeast vector YEp13, named pTN1171. (B) The synthetic DNA oligomer was constructed using the DNA synthesizer (Applied Biosystems 380A). 'Met' in the figure is placed on the start ATG of α-factor prepro signal. The asterisk in the promoter region shows the point mutation introduced to make a new *Pvu*II site in the original *GAL7* promoter sequence. Other asterisks on the coding region indicate the positions of codon replacement from the original α-factor prepro sequence to the codons observed more frequently in *S. cerevisiae* (Bennetzen and Hall, 1982).

mnn (mannan synthesis) mutants defective in various stages of mannose outer chain addition were also isolated by Ballou's group (Ballou *et al.*, 1980; Ballou, 1982). Among them, only *alg1* and *alg2* mutants lack outer chains in glycoprotein and show a temperature-sensitive lethality. According to the idea that the outer chain elongation may be essential for cell growth, we have tried to isolate similar but different glycosylation-defective mutants by combining the isolation of temperature-sensitive (*ts*) mutants with the selection of the mutants which produce core-glycosylated invertase like *sec18* mutant. The *sec18* mutant is blocked in protein transport from the endoplasmic reticulum (ER) to Golgi at the non-permissive temperature (Novick *et al.*, 1980, 1981) and is believed to accumulate an oligosaccharide of $Man_8GlcNAc_2$, which is formed in the ER by the removal of three glucose units and a single α1,2-linked mannose unit from the common lipid-linked precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ (Byrd *et al.*, 1982; Esmon *et al.*, 1984; Haselbeck and Schekman, 1986).

Here we report the isolation and characterization of new mannose outer chain-deficient mutants (*och1* and *och2*), which contain the same or a slightly larger oligosaccharide than that of the *sec18* mutant at the non-permissive temperature, and have the potential advantages for the production of mammalian-derived glycoprotein, like human tissue-type plasminogen activator (tPA), without adding high mannose outer chains.

Table 1. Isolation of *och* mutants

	Duration of [^{3}H]mannose suicide	
	4 weeks	8 weeks
Initial recovery	120,000	7200
ts mutants	876	80
[^{3}H]Man/[^{35}S]Met selection*	64	Not done
Invertase staining†	1 (M5)	1 (M168)

*The mutants which exhibited normal incorporation of [^{35}S]methionine and reduced incorporation of [^{3}H]mannose at 36°C were selected.
†The mutants which made the invertase containing the same or slightly larger size of oligosaccharide than that of the *sec18* mutant at the non-permissive temperature were selected by activity staining of SDS–polyacrylamide gel electrophoresis as described in Materials and Methods.

MATERIALS AND METHODS

Strains and genetic methods

The parental strain *S. cerevisiae* EHA-1C (*MAT***a** *leu2-3 leu2-112 pep4-3*) was obtained through genetic crosses of three yeast strains, X-2180-1A (*MAT***a** *SUC2 mal mel gal2 CUP1*), DBY746 (*MAT*α *his3-Δ1 leu2-3 leu2-112 ura3-52 trp1-289a*) and 20B-12 (*MAT*α *pep4-3 trp1*), which were obtained from the Yeast Genetic Stock Center. KD115 (*MAT*α *ole1*), X10-1C (*MAT***a** *rad6-1 ade2-1*), X10-2A (*MAT*α *rad6-1 ade2-1*), IS428-36B (*MAT*α *lys5 cyh2*) and KS80-10B (*MAT***a** *och1 cyh2 pep4 can1 ura3 hom3 leu2*) were also used for genetic analysis. HMSF2176 (*MAT***a** *sec18-1*) obtained from the Yeast Genetic Stock Center was used as a typical *sec18* mutant. The *mnn9* mutant, LB347-1C, originally reported by Tsai *et al.* (1984), was kindly provided by Dr W. Tanner (University of Regensburg, Germany), and was used to examine the complementation between *och1* and *mnn9*. One of the segregants designated as EHF-2C is a typical *och1* mutant used in this study. Yeast genetic methods were described by Mortimer and Howthone (1975). Genetic mapping of mutations in *S. cerevisiae* was performed according to Sherman *et al.* (1986). Yeast transformation was by the lithium acetate method (Ito *et al.*, 1983). Recombinant DNA manipulation was as described (Maniatis *et al.*, 1982).

Isolation of mutants

The [^{3}H]mannose suicide selection was used to isolate the mutants according to the report of Huffaker and Robbins (1982). Briefly, the parental strain EHA-1C grown in YEPD medium (1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose) was mutagenized with 0·5% $NaNO_2$, grown in YEPD medium containing [^{3}H]mannose at 36°C for 30 min after 1 h preincubation at 36°C, and kept at −80°C for 4 or 8 weeks. Then the surviving cells were plated on YEPD and grown at 25°C. The candidates for *ts* mutants were screened for the accumulation of low molecular weight secretory form of invertase at 36°C by active staining on polyacrylamide gel, after with or without pre-screening for the smaller ratio of [^{3}H]mannose incorporation relative to [^{35}S]methionine. For the detection of invertase, gels were bathed in 0·1 M-sucrose and 0·1 M-sodium acetate, pH 5·1, at 37°C for 20 min, washed with water, and then placed in 0·1% 2,3,5-triphenyltetrazolium chloride and 0·5 M-NaOH and boiled to develop red bands (Huffaker and Robbins, 1982).

Cell viability and microscopic analysis

A portion of culture was removed at a time indicated and briefly sonicated to dissociate cell clumps (sonication 0·5 s × 4 at strength 3, Sonifier 350, Tomy Seiko Co. Ltd, Japan). The cell suspension was divided into two parts; one was appropriately diluted and spread on YEPD plates to measure the number of viable cells and the other was fixed with an equal volume of ethanol and examined under the microscope to determine total cell number and frequency of unbudded cells.

Preparation of invertase

EHF-2C (*MAT***a** *och1 leu2-3 leu2-112 pep4-3*) was grown in a 5-l jar fermenter containing 3 l of YEPD medium with 0·3 M-sorbitol at 20°C. When glucose concentration monitored by glucose analyzer (Model PM-1000, Toyo Jyozo Co. Ltd, Japan) reached less than 0·02%, 0·5% sucrose was added to induce the invertase gene expression and the temperature was either kept at 20°C or increased to 36°C. The cells were incubated for an additional 5 h at each temperature and then harvested. Invertase was purified by the method of Neumann and Lampen (1967) with some modifications. The cells obtained from 12-l cultures were broken by French pressure cell press (SLM, Aminco) at 12,000 psi and the homogenates were centrifuged to remove the cell debris. Ammonium sulfate was added to the supernatant until 75% saturation to precipitate most of

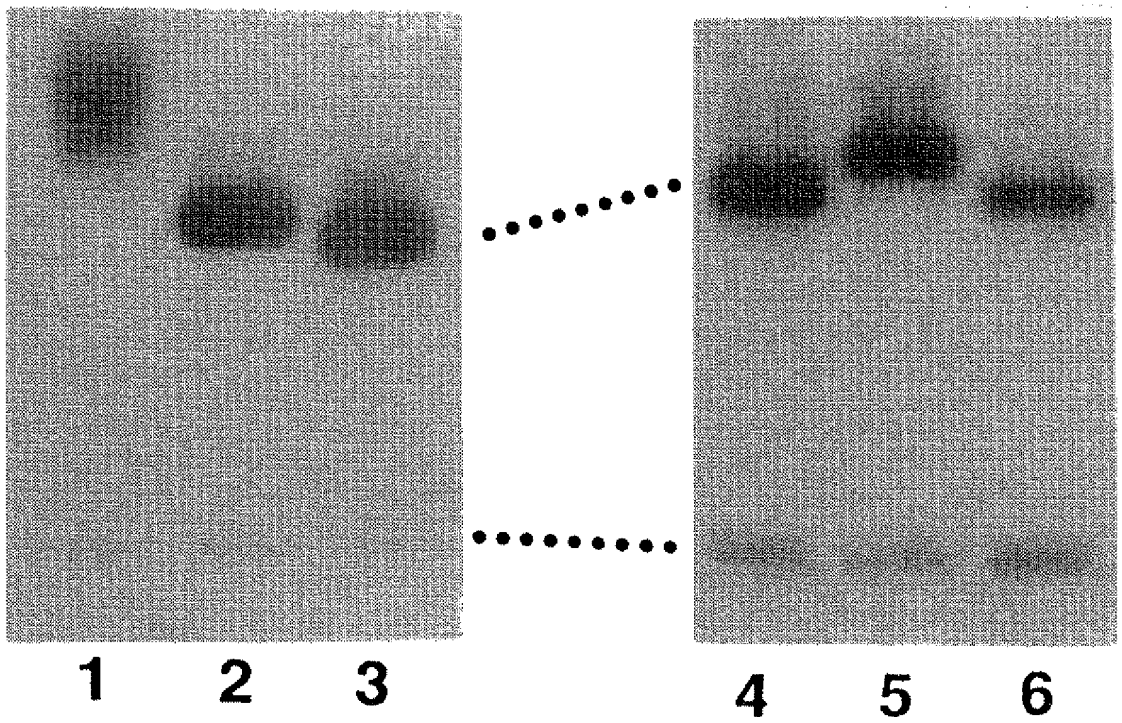

Figure 2. Difference in the migration of invertase among the mutants. The pattern of the oligosaccharide chain was examined by invertase staining gel. The lysates were prepared from the cells cultured for 1·5 h at the non-permissive temperature as follows: the cells were washed twice with a buffer containing 50 mM-Tris–HCl (pH 7·4), 1 mM-EDTA, 1 mM-EGTA, 1 mM-DTT, 0·1 mM-PMSF, 0·2 mM-DIFP, 10 μg/ml leupeptine, and 1 μg/ml aprotinin, and resuspended in the same buffer. Glass beads (0·45–0·50 mm in diameter) were added up to the surface of the cell suspension, and vortexed for 1 min with cooling down on ice. After adding an equal volume of buffer and spinning in a microcentrifuge for 5 min, the supernatant was used as a lysate. After 5% SDS–polyacrylamide gel electrophoresis, invertase active staining was done according to the procedure described in Materials and Methods. Lane 1, EHA-1C strain (wild type); lanes 2 and 5, EHF-2C strain (*och1*); lanes 3 and 4, M5 strain (*och2*); and lane 6, HMSF176 strain (*sec18*). The secretory form of invertase of the *och1* mutant had a slightly lower mobility than that of the *sec18* mutant, while the *och2* mutant exhibited the same mobility as that of *sec18* on the gel.

the proteins including a non-glycosylated cytosolic form of invertase. After centrifugation, the supernatant containing glycosylated cell surface invertase was dialyzed against 10 mM-potassium phosphate buffer, pH 6·5. DEAE–Sephadex A-50 resin equilibrated in advance with the buffer was added to the dialysate, then the resin was packed in a column (2·6 × 35 cm) and washed with 2 volumes of the same buffer containing 0·1 M-NaCl. Invertase was then eluted with the buffer containing 0.2 M-NaCl and the fractions exhibiting enzyme activity were pooled and lyophilized. This crude sample was dissolved in deionized water and loaded onto a Sephadex G-200SF column (2·0 × 95 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 6·5, containing 0·2 M-NaCl. The column was eluted with the same buffer and enzyme fractions were pooled and lyophilized.

Analysis of N-linked oligosaccharide length of invertase

The purified cell surface invertase (0·2 mg) was subjected to hydrazinolysis and N-acetylation according to Takasaki *et al.* (1982). The released N-linked oligosaccharide was pyridylaminated by using Pyridylamination Reagent Kit (Takara, Japan). The pyridylaminated (PA)-sugar was passed through a TSK-gel HW-40F column (1·0 × 40 cm) and detected by fluorescence (excitation wavelength (Ex) = 320 nm, emission wavelength (Em) = 400 nm). The length of PA-sugar was separated on a Glyco-Pak N (Waters, 0·78 × 30 cm) at a flow rate of 1·0 ml/min. Two solvents, A and B, were used. Solvent A was a mixture of acetonitrile–3% (v/v) aqueous acetic acid (pH 7·3 by triethylamine) (7:3) and solvent B was the same mixture in a

Table 2. Efficiency of invertase secretion at the non-permissive temperature

Strain	Genotype	Activity 25°C	Activity 36°C	Ratio (%) (36°C/25°C)
EHA-1C	Wild type	0·042	0·025	59·5
EHF-2C	*och1*	0·130	0·132	101·5
M5	*och2*	0·040	0·024	60·0
HMSF176	*sec18*	0·054	0·0048	8·9

Each strain was cultivated for 16 h in YEPD medium at 25°C, then separated into two portions and incubated for an additional 30 min at either 25 or 36°C. The cells were precipitated by centrifugation and resuspended in glucose-starved YEPD medium containing 0·05% glucose and cultured for an additional 1·5 h at 25 or 36°C to derepress the invertase gene expression. These cells were precipitated after addition of 20 mM-sodium azide. The cell pellets were resuspended in 0·5 ml of 200 mM-phosphate buffer (pH 7·0) and incubated for 20 min at 30°C after addition of 0·2 ml of 0·5 M-sucrose. The reaction was terminated by boiling for 3 min. The cells were removed and the product in the supernatant was determined by the method of Somogyi (1952). The activities represent the OD_{660} values of Nelson-Somogyi's reaction divided by the cell density (OD_{600}).

Table 3. Frequency of unbudded cells after the temperature shift

Strain (genotype)	Temperature (°C)	Frequency of unbudded cells (%) 0	6	10 (h)
EHF-2C	25	34	35	34
(*och1*)	36		13	13
M5	25	32	30	28
(*och2*)	36		22	18
EHA-1C	25	42	39	41
(wild type)	36		41	77

The experimental procedure is the same as in Figure 5.

ratio of 3:7. The column was first equilibrated with solvent A and 25 min after injection, the proportion of solvent B was linearly increased to 20% within 35 min, then to 100% within an additional 15 min. PA-sugar chains were detected by fluorescence (Ex = 310 nm, Em = 380 nm). PA-glucose oligomer (G_4-G_{20}, Honen Corporation, Japan) was used as a standard for size estimation.

Plasmid constructions

The cDNA clone encoding human tPA was obtained from Zymogenetics Inc. as the tPA expression vector pDR1496. Plasmid pYF1016 containing the *GAL7* promoter was provided by Prof. T. Fukasawa (Nogi and Fukasawa, 1983; Tajima *et al.*, 1985). The galactose-inducible tPA expression vector pTN1171 is shown in Figure 1A. The whole expression unit (*GAL7* promoter, α-factor prepro, tPA cDNA and *TPI* terminator) was introduced in the unique *Bam*HI site in YEp13. The *GAL7* promoter was derived from the *Dra*I- and *Sfa*NI-digested 592 bp fragment of pYF1016. The promoter and α-factor 5′ end regions were fused to the synthetic linker shown in Figure 1B. The rest of the expression unit (α-factor prepro region, tPA cDNA and *TPI* terminator) was derived from the *Pst*I-*Bam*-HI fragment of pDR1496.

RESULTS

Isolation of the mutants

Two different experiments were performed to isolate mutants (Table 1). The first experiment involved a 4-week stock of mutagenized cells at −80°C after incorporation of [^{3}H]mannose, a selection of *ts* candidates, a pre-screening step to confirm that there was less incorporation of [^{3}H]mannose relative to [^{35}S]methionine, followed by the analysis of invertase mobility (Huffaker and Robbins, 1982). The second experiment involved a longer 8-week stock of mutagenized cells to enrich the mutant populations which escaped the [^{3}H]mannose suicide, and omitted the pre-screening process described above, because the first experiment may miss the mutants which incorporate [^{35}S]methionine insufficiently due to either the lethality of cells or the lack of *de novo* protein synthesis after shifting up the growth temperature from 25 to 36°C. In the first experiment, 876 *ts* mutants were selected from the survival pools of about 120,000 cells. The 64 *ts* candidates were selected from 876 *ts* mutants by the lesser incorporation of [^{3}H]mannose relative to [^{35}S]methionine and finally among them was isolated one *ts* mutant called M5, which showed a higher mobility of glycosylated form of invertase at the non-permissive temperature than others and wild-type cells. In the second experiment, 80 *ts* candidates were obtained from the survival pools of about 7200 cells. Finally, among them, one *ts* mutant called M168 was isolated through the direct analysis of invertase by gel electrophoresis.

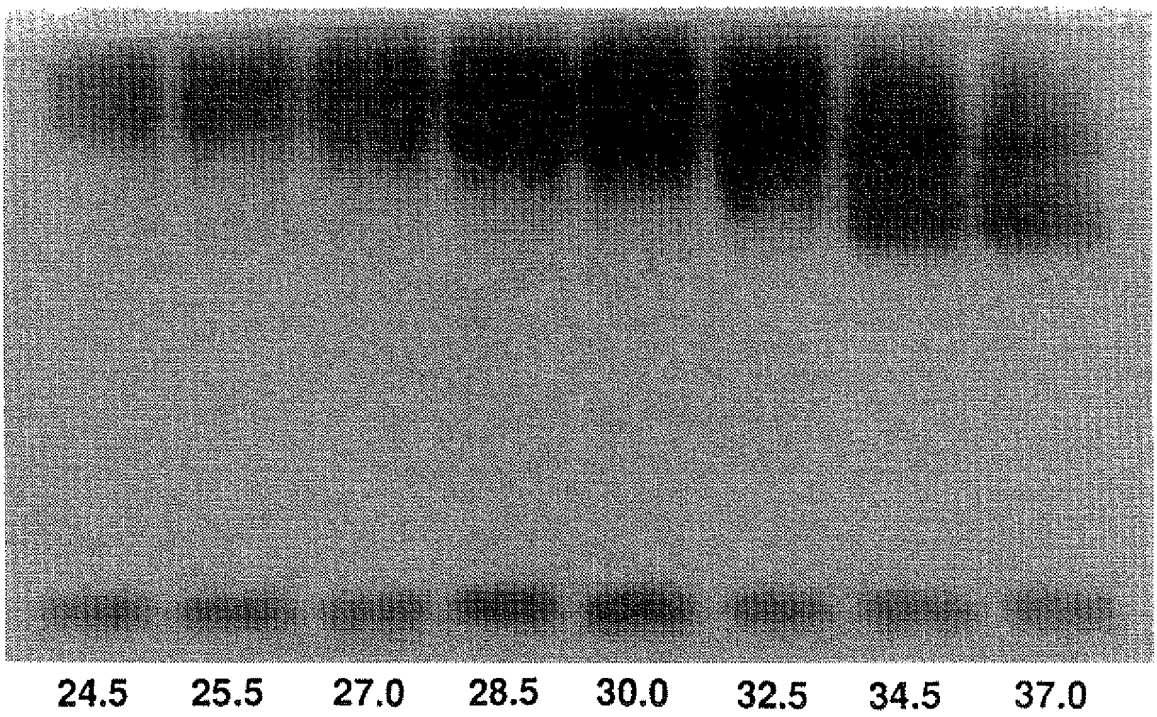

Figure 3. The temperature dependency of invertase migration in the *och1* mutant. The cells were cultured at the various temperatures as indicated. Each lysate was prepared as described in Figure 2. Electrophoresis was performed with 5% SDS–polyacrylamide gel according to the method of Laemmli (1970). Active staining of the gel was described in Materials and Methods.

Figure 2 shows the electrophoretic pattern of invertase after active staining of the gel. To isolate the new mutants which show different properties from the know *alg* mutants, we compared the size of the glycosylated form of invertase in the mutants with that in the *sec18* mutant, because all the *alg* mutants indicating the *ts* phenotype for cell growth are known to accumulate a much smaller oligosaccharide $GlcNAc_2$, or $Man_{1-2}GlcNAc_2$, or dispersed size of $Man_{1-8}GlcNAc_2$ at the non-permissive temperature (Huffaker and Robbins, 1983). While the M5 strain showed an identical mobility of invertase to that of the *sec18* mutant, the M168 strain showed a slightly slower mobility than the *sec18* mutant, suggesting that these mutants lack the polymannose outer chain at the higher temperature. After crossing with various wild-type cells, both diploid strains showed normal growth at the non-permissive temperature, indicating a recessive mutation. The M5 strain formed spores in less than 0·1% of diploid cells under the experimental conditions, presumably due to the additional dominant mutation on sporulation. In contrast, the M168 strain showed a 2+:2− segregation with regard to the temperature sensitivity in 200 asci. The linkage between the temperature sensitivity and the mannose outer chain deficiency was confirmed by the cosegregation of two phenotypes in 20 typical asci tested (data not shown). These results indicated that at least the M168 strain involves a single recessive gene mutation. The M168 strain was back-crossed three times with the parental strain to analyse the mutation. The *ts* phenotype of a segregant EHF-2C derived from the M168 strain was complemented by crossing with the M5 strain, suggesting that the mutations in both strains are different. Therefore, we designated the M168 and M5 mutation as *och1* and *och2* (outer chain elongation), respectively.

To exclude the possibility that the sugar chain deficiency may be derived from the mutation which blocks the secretory pathway like the *sec18*, we analysed the efficiency for invertase secretion in the *och* mutants at the non-permissive temperature. As shown in Table 2, the EHA-1C strain (wild-type cells) secreted 59·5% of invertase at 36°C as efficiently as at 25°C. Whereas the *sec18* mutant was deficient in the secretion of invertase at 36°C (8·9% of that at 25°C), the EHF-2C strain (*och1*) and M5

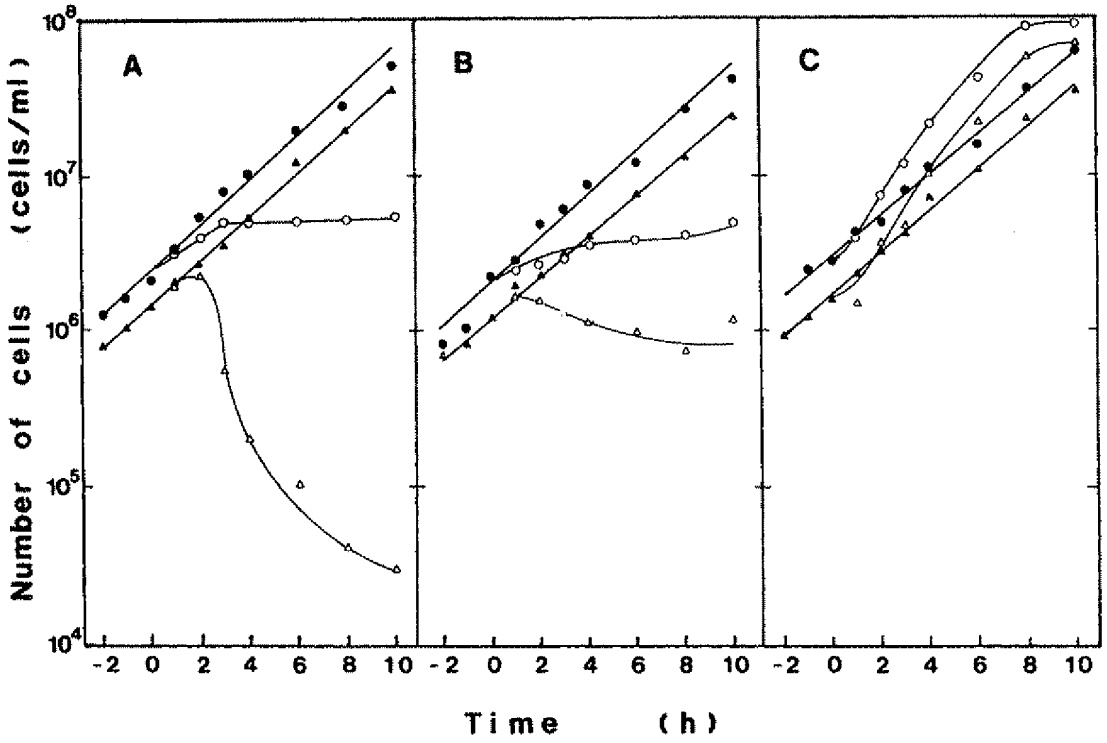

Figure 4. Growth curve and viability of cells after shifting the temperature. Cells of strain EHF-2C (A), M5 (B) and EHA-1C (C) growing in YEPD medium at 25°C were divided into two equal parts. One portion was kept in the same growth condition, and the other portion was shifted up to a temperature of 36°C at time zero. Cell number was measured in a hemocytometer and viability was determined by colony formation as described in Materials and Methods. (●) Number of total cells at 25°C; (○) number of total cells at 36°C; (▲) number of viable cells at 25°C; and (△) number of viable cells at 36°C.

strain (*och2*) secreted invertase at 36°C as efficiently as at 25°C (101·5% and 60·0%, respectively). These results indicated that the *och* mutants were not analogous to a series of *sec* mutants.

Figure 3 shows the temperature dependency of invertase migration on the SDS–polyacrylamide gel. While the wild-type strain EHA-1C did not change the invertase migration by the temperature shift (data not shown), the *och1* mutant EHF-2C represented the temperature dependency of migration, indicating the thermosensitive defect in a certain part of the mannose outer chain elongation pathway. The similar temperature dependency of invertase mobility was also observed in the *och2* mutant (data not shown).

Physiological properties of och *mutants*

The above results demonstrated that the *och* mutants were temperature sensitive for protein glycosylation and cell growth. To investigate the properties of *och* mutants in more detail, we examined the cell growth and colony-forming activity due to the temperature shift from 25 to 36°C (Figure 4). Mutant strains EHF-2C (*och1*) and M5 (*och2*) and wild-type strain EHA-1C were grown to mid-logarithmic stage at the permissive temperature (25°C), and shifted up to the non-permissive temperature (36°C). Increase in cell numbers of the *och1* mutant stopped after 3–4 h at the non-permissive temperature and the cell number did not double after the temperature shift even in the prolonged cultivation. Colony-forming activity was rapidly decreased; only a few per cent of cells survived at 10 h after shifting up the temperature (Figure 4A). Colony-forming activity of M5 strain (*och2*) was not decreased significantly, more than 25% of cells could survive after 10 h of incubation at the non-permissive temperature, while the total cell number profile was the same with that of EHF-2C strain (*och1*) (Figure 4B).

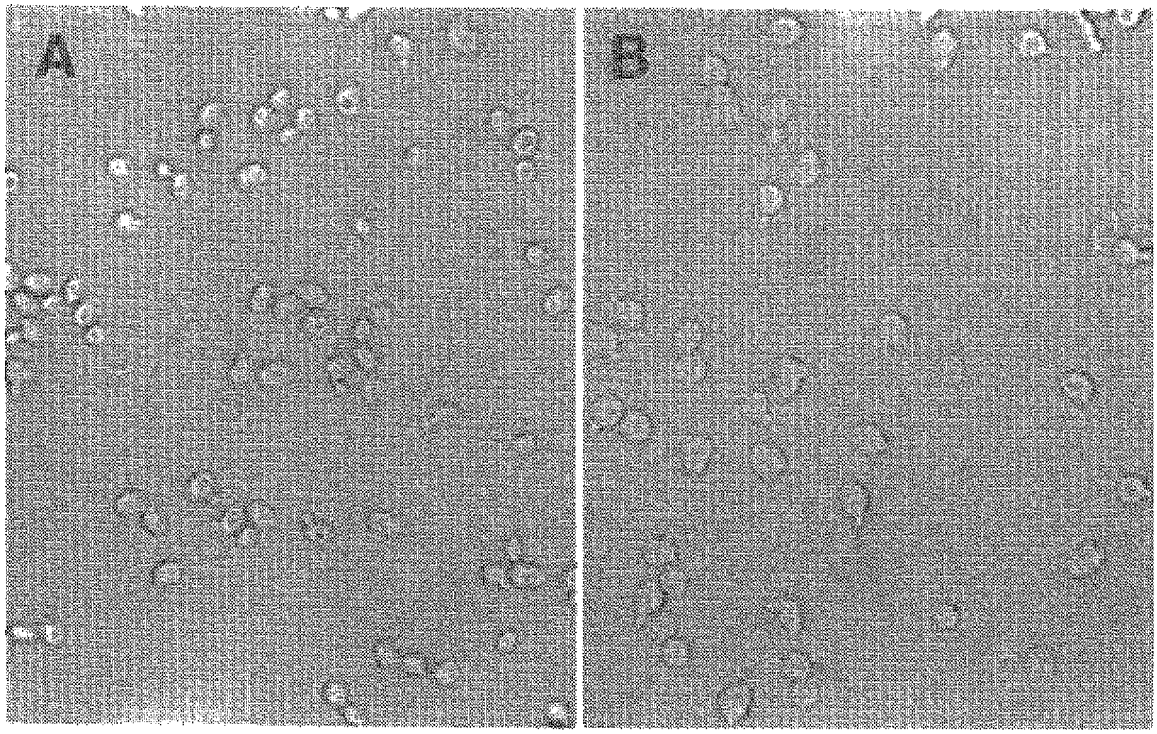

Figure 5. Cell morphology of the *och1* mutant. Cells of strain EHF-2C were grown in YEPD medium at 25°C and divided into two portions. One of them was shifted up to a temperature of 36°C, followed by incubating for 6 h. The cells were briefly sonicated, fixed with 50% ethanol, and photographed under the microscope. (A) Cells incubated for 6 h at 25°C; (B) cells incubated for 6 h at 36°C.

Morphology of cells cultivated at the non-permissive temperature was examined under the microscope to determine whether or not the cells would cease growth at a specific stage of the cell cycle. If the cells stopped at the G1 phase of the cell cycle, most of their terminal phenotype would result in the unbudded morphology. In contrast, if the cells stopped at a stage other than the G1 phase, their phenotype would result in the budded morphology (Hartwell *et al.*, 1974). In *och1* mutant cells, the per cent of unbudded cells was decreased from 35% at 25°C to 13% after incubating at 36°C for 6 h (Table 3). Together with the result that the ratio of unbudded cells for a 10-h incubation at 36°C remained constant (13%), the above data suggested that the cell growth of the *och1* mutant will be arrested at a stage other than the G1 phase. The terminal phenotype of the *och1* mutant was further examined by microscopy. It is noteworthy that the cells have a uniformly small-budded shape like a certain *cdc* mutant (Hartwell *et al.*, 1973) (Figure 5B), suggesting that the *och1* mutant cells stop their growth at a specific point in the early stage of bud formation.

Genetic mapping of OCH1 *gene*

We mapped the *och1* mutation genetically on the yeast chromosome. In the preliminary experiment, we found that the *och1* mutation is linked with *cyh2* on chromosome VII. Furthermore, as shown in Table 4, the detailed assignment of this gene was performed with some markers located near the *cyh2* site. From the results of tetrad analysis, the *och1* mutation is mapped at 32·4 cM from *cyh2*. The *lys5* mutation is also located 33·5 cM distal from *cyh2*, but not tightly linked with *och1* (61·4 cM), and *och1* is weakly linked with the centromere (57·8 cM). Therefore, the *och1* mutation is mapped on the proximal side of *cyh2* at a distance of 32·4 cM. The *ole1* and *rad6* mutations are known to be located in this region of chromosome VII (Mortimer *et al.*, 1989). We attempted to use the *rad6* mutant to map the *och1* mutation. However, at least in the cross between strains YS37-11C and X10-1C, no linkage was recognized between *rad6* and *och1* or *cyh2*, indicating that *rad6*, at least in the strains used in this study, does not reside on this region of chromosome VII. In contrast, *och1* is found to be tightly linked

Table 4. Tetrad analysis of the *och1* mutant

Interval	Ascus type PD	T	NPD	Distance (cM)
*och1-cyh2**†	226	340	5	32·4
*och1-lys5**†	122	391	50	61·4
*cyh2-lys5**†	246	321	11	33·5
och1-CEN*	58	159	58	57·8§
och1-rad6†	39	184	57	93·9
cyh2-rad6†	44	189	54	89·4
och1-ole1‡	13	3	0	—¶
och1-cyh2‡	9	7	0	—¶
cyh2-ole1‡	6	10	0	—¶

The intervals, *och1-cyh2*, *och1-lys5* and *cyh2-lys5*, were expressed with the sum of the results in the crosses EHF-2C × IS428-36B and YS37-11C × X10-1C. The three classes of tetrads are represented by PD (parental ditype), NPD (non-parental ditype) and T (tetra-type).

*EHF-2C (*MATa och1 leu2-3 leu2-112 pep4-3*) × IS428-36B (*MATα lys5 cyh2*).

†YS37-11C (*MATα och1 lys5 cyh2*) × X10-1C (*MATa rad6-1 ade2-1*). YS37-11C is a segregant derived from the cross EHF-2C × IS428-3B.

‡KS80-10B (*MATa och1 cyh2 pep4 can1 ura3 hom3 leu2*) × DK115 (*MATα ole1*).

§*leu2* was used as a centromere (CEN) marker. The centromere distance was calculated on the basis of its second-division segregation frequency.

¶Distance was not calculated due to the poor viability of spores, as described in the text.

with *ole1*, as judged from the lack of appearance of non-parental ditype (Table 4). However, as the spore viability of the cross KS80-10B × KD115 was very low, it is difficult to establish the genetic distance between *och1* and *ole1*. In the three-point test involving *cyh2*, *ole1* and *och1*, no recombinations occurred between *ole1* and *cyh2–och1* linkage, indicating the central location of *ole1* in these three genes (data not shown). Together with the alignment of these genes (*cyh2–ole1–och1*), the map for the relevant markers in this region is summarized in Figure 6. These data demonstrate that *och1* is a new gene which has not been mapped so far.

Analysis of N-linked oligosaccharide length of invertase

To confirm that the *och1* mutant is deficient in the mannose outer chain elongation at the non-permissive temperature, the length of N-linked oligosaccharide of the cell surface invertase was analysed by high pressure liquid chromatography. It was reported that the cell surface invertase contains 14 potential N-glycosylation sites, and of these 13 are wholly or partially glycosylated to give an average of nine to ten oligosaccharides/subunit (Reddy *et al.*, 1988). Among them 28·5% are larger than $Man_{50}GlcNAc_2$ and the residual 71·5% of total oligosaccharides consist of short chains ranging from $Man_8GlcNAc_2$ to $Man_{14}GlcNAc_2$ (Ziegler *et al.*, 1988). We confirmed the same distribution ($>Man_{50}GlcNAc_2 = 29\%$ and $Man_{8-14}GlcNAc_2 = 71\%$) in the invertase from wild-type baker's yeast (Boehringer Mannheim, Germany) (Figure 7A). The similar sugar chain profiles were also observed in the invertase from EHF-2C (*och1* mutant) grown at 20°C (Figure 7B). However, the ratio of oligosaccharides larger than $Man_{50}GlcNAc_2$ in the *och1* mutant is decreased from 27 to 7% and that of oligosaccharides smaller than $Man_{14}GlcNAc_2$ is increased from 83% to 93% in return, by shifting up the incubation temperature from 20 to 36°C (Figure 7C). It is noteworthy that the relative amount of $Man_{9-10}GlcNAc_2$ is remarkably increased in the *och1* mutant at the non-permissive temperature. These data clearly demonstrate that the mannose outer chain addition is blocked and the smaller oligosaccharide, especially $Man_{9-10}GlcNAc_2$, is accumulated in the *och1* mutant cells at the non-permissive temperature. It is most likely that the incomplete disappearance of the high mannose fraction ($>Man_{50}GlcNAc_2$) (Figure 7C) may be derived from the production of invertase containing high mannose outer chains due to the decrease of glucose concentration prior to the temperature shift, although the leaky phenotype of the *och1* mutant may not be excluded completely.

Human tPA expression in och1 *mutant*

Recent progress of yeast genetic engineering made it possible to use the yeast system for the production of heterologous proteins at the industrial level. But some problems still remain to be elucidated. One of them is the hyperglycosylation of the heterologous protein products. We tried to use the *och1* mutant EHF-2C as a host strain for the expression of mammalian glycoprotein, tPA. The tPA cDNA was fused to the α-factor prepro region for the secretion, and the inducible *GAL7* promoter was used for the expression. The *GAL7* promoter system is useful because it is one of the strongest inducible promoters in yeast and its expression can almost completely shut off in the absence of galactose.

As shown in Figure 8, the mutant strain EHF-2C produced a larger amount of fibrinolytically active

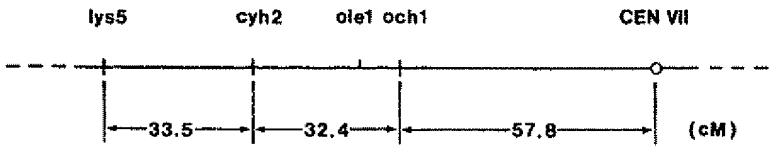

Figure 6. Genetic mapping of the *OCH1* gene.

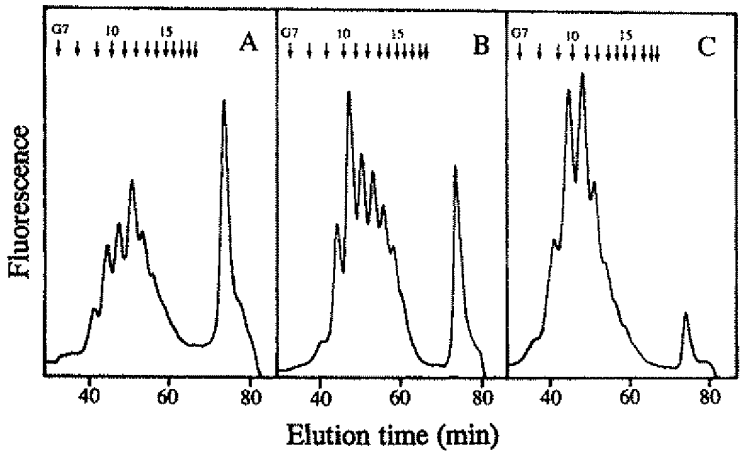

Figure 7. N-linked oligosaccharide length of invertase prepared from cell extract of *och1* mutant incubated either at 20°C (B) or at 36°C (C), in addition to that of invertase from wild-type baker's yeast (A). Analytical conditions were described in Materials and Methods. Arrows show the elution time of PA-glucose oligomers (G_7-G_{19} respectively).

tPA inside the cell than that from the parental strain EHA-1C. However, no tPA was detected in the medium, even from the mutant culture, either by the fibrinolytic assay or by the enzyme immunoassay. The mobility of tPA from EHF-2C incubated at the non-permissive temperature was the same as that from the mammalian cells (data not shown), indicating that the hypermannosylation of tPA was blocked in the mutant. These observations suggest that the inhibition of hyperglycosylation may cause the increased production of enzymatically active tPA inside the cell, although the poor secretion of tPA may not be improved by the lack of mannose outer chain.

DISCUSSION

In this study, we have isolated two mutants, *och1* and *och2*, which show both a deficiency in the elongation of mannose outer chain on glycoprotein and a temperature sensitivity for cell growth. The *och1* is a nuclear recessive gene mutation, located on the left arm of chromosome VII. In *och* mutants, the electrophoretic mobility of the glycosylated form of invertase is the same or slightly higher than that of the *sec18* mutant (Figure 2), suggesting that *och1* and *och2* mutants produce almost the same size of oligosaccharides as $Man_8GlcNAc_2$, which is believed to accumulate in the *sec18* mutant at the non-permissive temperature (Byrd *et al.*, 1982; Esmon *et al.*, 1984; Haselbeck and Schekman, 1986). Further, the lack of outer chain elongation in the *och1* mutant was confirmed by the lesser amount of N-linked high mannose oligosaccharide on cell surface invertase prepared from whole-cell extract of *och1* cells incubated at 36°C compared with that from *och1* cells incubated at 20°C or that from commercial baker's yeast (Figure 7).

Several researchers have reported the isolation of yeast mutants which showed a deficiency in protein glycosylation pathways. The *alg* mutants are deficient in a certain step of the biosynthetic pathway for the lipid-linked oligosaccharide, which is engaged in the ER (Huffaker and Robbins, 1982, 1983). Among the nine complementation groups (*alg1–alg8* and *gls1*), three complementation groups, *alg1*, *alg2* and *alg4*, showed a *ts* phenotype, but the other six did not. In addition, it is reported

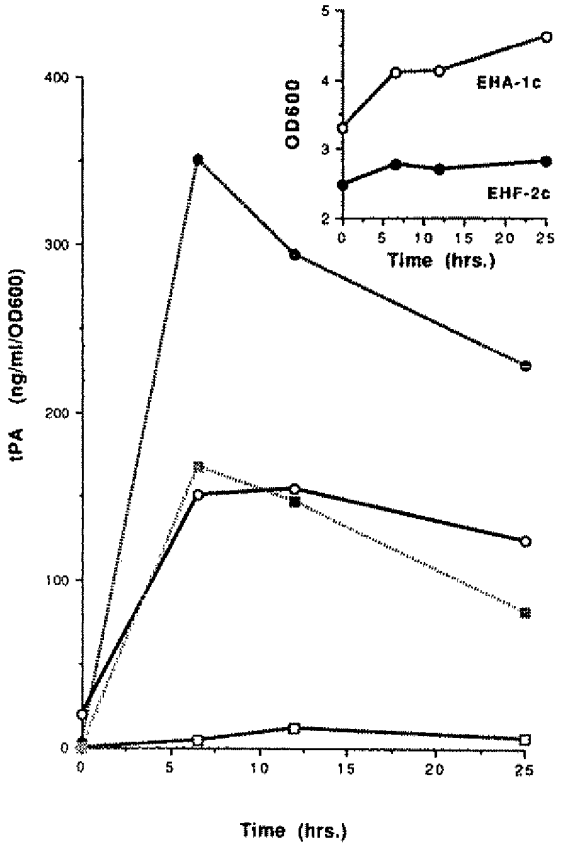

Figure 8. Time course of tPA expression. Each strain was cultured at 25°C for 16 h in YEPD medium, after washing the cells once with distilled water; the pellets were then resuspended in the glucose-starved medium containing 0·1% glucose and 4% galactose. The cells were cultured for an additional 25 h at 36°C. The cell pellets were washed once with phosphate-buffered saline (Dulbecco) and resuspended in 0·5 ml of 0·5 M-ammonium bicarbonate, then homogenized by agitation in the presence of glass beads (40 mesh size) using a vortex mixer (3 min, 5 times, intervals with intermittent cooling ice). After addition of 0·5 ml of 0·5 M-ammonium bicarbonate, cell debris was pelleted by centrifugation and protein supernatants were assayed. The active tPA was quantified by fibrinolytic assay using the fibrin plate (Hokken Co. Ltd) and total amount was estimated by enzyme immunoassay (EIA). (○) Amount of tPA in EHA-1C estimated by EIA; (●) amount of tPA in EHF-2C estimated by EIA; (□) amount of active tPA in EHA-1C estimated by fibrinolytic assay; and (■) amount of active tPA in EHF-2C estimated by fibrinolytic assay. The inset represents the cell density profile of EHA-1C (○) and EHF-2C (●) during the experiment.

that the small oligosaccharide chains such as $GlcNAc_2$ or $Man_{1-2}GlcNAc_2$, which accumulate in the *alg1* and *alg2* mutants, respectively, at the non-permissive temperature, do not serve as substrates for outer-chain elongation (Huffaker and Robbins, 1983). The other *ts* mutant *alg4* accumulated multiple oligosaccharides ranging from $Man_1GlcNAc_2$ to $Man_8GlcNAc_2$, but these oligosaccharides attached to invertase were not elongated by the addition of mannose outer chain (Huffaker and Robbins, 1983). In contrast, the other *alg* mutants, which can grow even at 36°C, accumulated the lipid-linked oligosaccharide chains larger than $Man_5GlcNAc_2$, which are utilized as substrates for further elongation in the Golgi apparatus.

The *och1* mutant shows the *ts* phenotype similar to some *alg* mutants (*alg1*, *alg2* and *alg4*). However, the terminal phenotypes at the non-permissive temperature are different between *och1* and *alg* mutants. The *och1* mutant stops its growth in the small-budded morphology (Figure 5), while the *alg2* mutant stops at a random phase of the cell cycle (Huffaker and Robbins, 1983) and the *alg1* mutant stops at the G1 phase (Arnold and Tanner, 1982; Klebl *et al.*, 1984). Further, the mobility of invertase in the *och1* mutant is different from that in *alg* mutants at the non-permissive temperature (Figures 2 and 3, and Huffaker and Robbins, 1983). The analysis of oligosaccharide length in invertase accumulated in the *och1* mutant at 36°C clearly demonstrated the increase of smaller oligosaccharides ($Man_{9-10}GlcNAc_2$) and the decrease of larger oligosaccharides ($>Man_{50}GlcNAc_2$), as compared with that from the *och1* mutant grown at 20°C or that from commercially available invertase prepared from wild-type baker's yeast (Figure 7). These data indicate that the *och1* mutant is different from the known *alg* mutants.

The *mnn* mutants show a deficiency in the elongation of polymannose outer chain (Ballou, 1982). Among the *mnn* mutants reported so far, only the *mnn9* resembles the *och1* in the size of glycosylated invertase. Different from *och1* mutant, it is reported that the mannan-defective phenotype of the *mnn9* mutant does not cosegregate with the *ts* phenotype (Ballou *et al.*, 1980). We have compared the *och1* and *mnn9* mutants. The outer chain-defective phenotype of the *och1* mutant was complemented by the cross with the *mnn9* mutant, showing the hypermannosylated form of invertase (data not shown). Accordingly, it is clear that the *och1* mutation is not allelic to the *mnn9* mutation.

Another mutant which shows a similar phenotype to *och1* is *ssc1*, originally isolated by its supersecretion of several foreign proteins (Smith *et al.*, 1985). Proteins secreted from the *ssc1* mutant also lack the mannose outerchain. The *scc1-1* mutant showed the same mobility of invertase as that of

the *mnn9* mutant, indicating the production of oligosaccharide structure of $Man_{10-11}GlcNAc_2$ (Rudolph *et al.*, 1989). *SSC1* has been found to be identical to *PMR1*, which is a member of a Ca^{2+} ATPase gene family and encodes 950 amino acids with eight transmembrane segments (Rudolph *et al.*, 1989). The map position of the *PMR1* gene is located close to *lys5* on chromosome VII, which is different from that of the *OCH1* gene described above.

As a conclusion, the above data strongly suggest that the *och1* mutation is a new mutation which has not been reported so far. There are three possibilities on the function of the *OCH1* gene. First, it may encode mannosyltransferase that initiates the elongation of the polymannose outer chain. It is noteworthy that Hernandez *et al.* (1989b) predicted the existence of new mutants which must be different from the *mnn9* mutant and also discussed the possibility that the mannose outer chain addition may be divided into two different steps, initiation of outer chain addition and subsequent elongation. If it is true, the *OCH2* and *OCH1* genes may correspond to the specific mannosyltransferases responsible for the initiation of mannose outer chain and subsequent elongation of outer chain, respectively. Second, the *OCH1* gene product may function in the biosynthesis of lipid-linked oligosaccharide like *ALG2*, because the pathway still contains sequential reactions which will be catalyzed by several unknown mannosyltransferases between the steps from $Man_3GlcNAc_2$-PP-Dol to $Man_9GlcNAc_2$-PP-Dol (Kukuruzinska *et al.*, 1987). Although the lipid-linked oligosaccharides larger than $Man_5GlcNAc_2$ are reported to become substrates for further elongation in the Golgi, the possibility may still remain that the abberrant lipid-linked oligosaccharides larger than $Man_5GlcNAc_2$ may not become substrates for outer chain elongation. Third, the *OCH1* protein may function in the protein transport either from ER to Golgi or inter-Golgi cisternae. The defect of the *OCH1* gene product may open a bypass of normal secretory pathways. Recently we have succeeded in the cloning of the *OCH1* gene by the complementation of the *ts* phenotype of the *och1* mutant. We are further addressing the above possibilities through the analysis of its gene product.

Despite the effort of many researchers, the production of mammalian glycoprotein by *S. cerevisiae* in large scale is not satisfactory. Several factors may influence the efficient production and secretion of foreign proteins in yeast, for instance: (1) efficiency of translation, (2) size of protein to be produced, (3) efficiency of protein folding, (4) proteolysis of foreign protein, (5) differences in protein transport, sorting and secretion system, and (6) presumably effects of hyperglycosylation. The level of tPA production which we achieved using the *och1* mutant was 100–200 ng/ml/OD, which was too low to investigate the pharmaceutical feasibility. In the expression of the modified tPA, whose N-terminal finger growth factor domain was truncated, a small but significant amount of tPA activity was detected in the medium only by using the *och1* mutant (data not shown). Neither native tPA nor the truncated form of tPA was secreted in the *OCH1* wild-type strain. These observations suggested that the *och* mutants might be more efficient in secreting the mammalian glycoprotein than the wild-type cells which produce a hyperglycosylated form of glycoproteins. Even so, the *och1* mutation alone is not sufficient for the production of heterologous glycoproteins. The combination of other mutations such as 'supersecretion' with the *och* mutation may serve for the production of mammalian glycoprotein like tPA in larger amounts at the industrial level.

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